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Comparison of Granule Proteins from Human Polymorphonuclear Leukocytes Which Are Bactericidal toward *Pseudomonas aeruginosa*

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Killing of *Pseudomonas aeruginosa* by a 55-kDa bactericidal protein (BP 55), a 30-kDa protein (BP 30), cathepsin G, elastase, and proteinase 3 has been compared. *P. aeruginosa* was resistant to killing by elastase and proteinase 3. BP 55 at a 50% lethal dose (LD_{50}) of 0.23 μg of protein per 5×10^6 bacteria per ml killed *P. aeruginosa* and was far more active than BP 30 and cathepsin G. The LD_{50}s of BP 30 and cathepsin G were 16.9 and 28.3 μg of protein per 5×10^6 bacteria per ml, respectively. Preincubation of BP 55 or BP 30 with lipopolysaccharide (LPS) from *P. aeruginosa* inhibited bactericidal activity. The N-terminal amino acid sequence of BP 55 and BP 30 revealed no relationship between the two proteins. However, a monoclonal antibody (AHN-15) reacted with both proteins by Western immunoblot. The bactericidal activity of cathepsin G toward *P. aeruginosa* appeared to be dependent on the availability of the active site of the enzyme; bactericidal activity was inhibited by phenylmethylsulfonyl fluoride (PMSF) and by the specific cathepsin G inhibitor, Z-Gly-Leu-Phe-CH_2Cl. The enzyme and bactericidal activities of cathepsin G were also inhibited by LPS from *P. aeruginosa*. LPS from *P. aeruginosa* was shown to be a competitive inhibitor of the enzyme activity of cathepsin G. Elastase enzyme activity was also inhibited noncompetitively by LPS, but the enzyme was not bactericidal. We have concluded that all three bactericidal proteins (BP 55, BP 30, and cathepsin G) may bind to the LPS of the outer membrane of *P. aeruginosa*. It appears that the enzyme active site must be available for cathepsin G to kill *P. aeruginosa* and that the active site may be involved in the binding of cathepsin G to *P. aeruginosa*.

Polymorphonuclear leukocytes (PMNL) occupy a central role in host defense and destroy intracellular microorganisms by both oxygen-dependent and oxygen-independent mechanisms. As reviewed by Spitznagel (45) and Thomas et al. (47), several proteins are implicated in oxygen-independent killing of microorganisms. Prominent among the cationic bactericidal proteins thus far purified from human PMNL is a 58- to 60-kDa bactericidal/permeability-increasing protein (B/PI) described by Weiss et al. (51). B/PI is bactericidal toward *Salmonella typhimurium* and *Escherichia coli*. Shafer et al. have described two cationic antibacterial proteins (CAP) of 57 kDa (CAP57) and 37 kDa (CAP37) that are bactericidal toward *S. typhimurium* (37). We have described a 55-kDa glycoprotein (BP 55) that was isolated from normal human PMNL and was bactericidal toward *Pseudomonas aeruginosa* (19, 20). The smaller cationic defensins (3.5 to 4.0 kDa) kill a spectrum of microorganisms, including *P. aeruginosa* (10, 16).

More recently, reports by Gabay et al. (9) and Campanelli et al. (6) of a 30-kDa protein, called azurocidin, which is bactericidal for *E. coli* and *Streptococcus faecalis* and fungicidal for *Candida albicans*, have generated a great deal of interest. These same investigators have recalled attention to the bactericidal activity of serine proteinases from PMNL, proteins which were among the earliest granule proteins analyzed for this biological activity (28, 29). Cathepsin G has been the most active serine proteinase in this regard, but enzyme activity was not required for killing of any of the bacteria studied thus far (1, 28, 38–41). We present evidence that BP 55 may be the same macromolecule as B/PI and CAP57. We report the purification of a 30-kDa protein (BP 30) which is bactericidal for *P. aeruginosa* and may be the same protein as azurocidin (9) and CAP37 (31). We also describe the enzyme-dependent bactericidal activity of cathepsin G toward *P. aeruginosa* and the interaction of this proteinase with lipopolysaccharide (LPS) from *P. aeruginosa*.

MATERIALS AND METHODS

Isolation of human PMNL. Venous blood was obtained in the form of leukocyte concentrates from the Red Cross, St. Paul, Minn. Erythrocytes were lysed in a two-step process involving ammonium chloride and hypotonic shock (20). Cytoplasmic granules were obtained by centrifugation as described previously (20), except that PMNL were lysed by nitrogen cavitation by the method of Borregaard et al. (5).

Purification of bactericidal protein and proteinases. BP 55 was purified from a granule extract by the two-column chromatography steps previously described (20). The entire peak of bactericidal activity from the second column, a Bio-Rex 70 cation-exchange column, was concentrated by ultrafiltration, adjusted to 0.15 M NaCl, and then applied to a reverse-phase C8 (Vydac) column (2.1 by 30 mm). Proteins were eluted by using a 20 to 70% (vol/vol) linear gradient of acetonitrile in 0.1% (vol/vol) trifluoroacetic acid developed over 45 min at a flow rate of one fraction per min. Fractions of 200 μl were collected, and up to 50 μl was used to screen for bactericidal activity after volatile solvents were eliminated under vacuum. To obtain purified native protein, we subjected the concentrated pool of bactericidal activity from

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the Bio-Rex 70 column to nondenaturing disc gel electrophoresis by the method of Thomas and Hodes (48) with a 20% polyacrylamide separating gel polymerized with ammonium persulfate. We applied 100 μg of partially purified protein to each disc gel (0.5 by 8 cm). Following electrophoresis, disc gels were sliced at 1.0-mm intervals with an electric gel slicer (model 195; Bio-Rad, Richmond, Calif.), and each slice was eluted in 0.01 N HCl at 4°C. Eluates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (22) and were analyzed for bactericidal activity as described below. The peak fractions of BP 55 and BP 30 were 13 and 7 mm, respectively, from the top of the gel. Eluates from gel slices 12 to 14 (BP 55) or 6 to 8 (BP 30) were pooled. Alternatively, native proteins were purified by molecular-sieving chromatography of 1.0 mg of protein of the concentrated pool of bactericidal activity from the Bio-Rex 70 column by using a Toyopearl HW 55S (TosoHaas Co., Philadelphia, Pa.) column (1.5 by 100 cm). Fractions (1.0 ml) were eluted in 0.05 M glycine buffer (pH 2.5) containing 0.5 M NaCl. Bactericidal fractions were characterized by SDS-PAGE (22), and peaks of eluted proteins were pooled. BP 55 was eluted in fractions 119 through 124, whereas BP 30 was found between fractions 135 and 155. After the major bactericidal peak was eluted from the first chromatography step, a dye-ligand affinity c lumn, the serine proteinases (elastase, cathepsin G, and proteinase 3) were collected as described elsewhere (21). Each of these proteinases was further purified by cation exchange with Bio-Rex 70 as previously described (21). The purity of the proteinases was assessed by silver staining of gels (27) following SDS-PAGE (22), by esterase staining (23) of nondenaturing gels (48) after electrophoresis, and by reactivity with specific substrates (21) as described below.

Protein was measured by the Hartree modification (13) of the Lowry method, with bovine serum albumin as the standard. SDS-PAGE was carried out in 5 to 20% gradient gels used in combination with a 3% stacking gel by the method of Laemmli (22). Protein bands were either visualized directly in the gel by silver staining (27) or electrophoretically transferred to nitrocellulose (Schleicher & Schuell, GIBCO Scientific, Coon Rapids, Minn.) after equilibration in a buffer consisting of 10 mM $NaHCO_3$, 3.0 mM Na_2CO_3, 0.1% SDS, and 10% methanol (7). Silver staining was done by the method of Oakley et al. (27) with an additional washing step in 0.005% citric acid before the color development. Prior to treatment with glycopeptidase F (Boehringer Mannheim, Inc., Indianapolis, Ind.), 3.0 μg of purified BP 55 or 6.0 μg of purified BP 30 was denatured as described by Campanelli et al. (6). One unit of glycopeptidase F was added, and the mixtures were incubated in a total volume of 30 μl at 37°C for 16 h before analysis by SDS-PAGE (22).

Bactericidal and proteinase assays. *P. aeruginosa* type I is immunotype 1 by the scheme of Homma (17) and is a clinical isolate from the University of Minnesota Hospitals, Minneapolis. It was maintained on blood agar plates and prepared for the bactericidal test as previously described (20). Proteins tested were incubated in optically matched tubes with 5×10^6 bacteria in a total volume of 1.0 ml of 0.08 M citrate phosphate buffer (pH 5.6) for 30 min at 37°C in a reciprocal shaking water bath. A 5-ml volume of nutrient broth (Difco Laboratories Inc., Detroit, Mich.) was added to the incubation mixture, and the surviving bacteria were allowed to grow to mid-logarithmic phase. The percent killing was determined by measuring the A_{650} and comparing that of bacteria preincubated with the proteins with that of bacteria preincubated in citrate phosphate buffer. Two additional tests were performed with each protein which appeared to be bactericidal. First, the A_{650} of both the test and control were measured at 30-min intervals, and it was observed that the extent of killing correlated with the length of the lag phase and that, once growth could be measured, the division times were identical. Second, standard dilution plate counts were performed at the end of the 30-min preincubation; this assay confirmed the results of the turbidimetric assay, showing that the effect of each protein was bactericidal rather than bacteriostatic. One bactericidal unit was defined as the amount of protein required to kill 95% of the 5×10^6 bacteria in the reaction mixture and was arrived at by dose-response in the turbidimetric assay, followed by linear regression analysis of the data.

The specific substrate assays for cathepsin G and elastase were done by using the chromogenic substrates Suc-Ala-Ala-Pro-Phe-pNA and Suc-Ala-Ala-Ala-pNA, respectively, as previously described (21). Methoxysuccinyl-Ala-Ala-Pro-Val-pNA was used as the substrate for proteinase 3 and as an alternative substrate for elastase. Suc-Ala-Ala-Pro-Phe-pNA was also used as the specific substrate for the activity of chymotrypsin (Sigma, St. Louis, Mo.). Inhibition of the enzyme activity of cathepsin G, elastase, proteinase 3, and chymotrypsin was quantitated after preincubation in enzyme buffer for 15 min at room temperature with the inhibitor before the addition of the specific substrate. The inhibitors used were dissolved in absolute ethanol and were 10^{-3} M phenylmethylsulfonyl fluoride (Sigma), 3×10^{-4} M Z-Gly-Leu-Phe-CH_2Cl (Enzyme Systems Products, Livermore, Calif.), and 5×10^{-4} M methoxysuccinyl-Ala-Ala-Pro-Val-CH_2Cl (Enzyme Systems Products). LPS from *P. aeruginosa* ATCC 27312 (List Biological Laboratories, Campbell, Calif.) or *Salmonella minnesota* (gift of Patrick Schlievert, University of Minnesota, Minneapolis) was suspended in 0.1% triethylamine. *P. aeruginosa* LPS is Fisher-Devlin immunotype 1, which is the same as Homma immunotype 8 (25).

Inhibition of bactericidal activity was tested by preincubation of the enzyme inhibitor or LPS with the granule protein in citrate phosphate buffer for 15 min at room temperature before the addition of 5×10^6 bacteria to the bactericidal assay. The percent killing of bacteria in control tubes with added inhibitor was subtracted before the percent inhibition was calculated. Killing of bacteria in control tubes was zero for LPS and did not exceed 5% with the other inhibitors. Ethanol never exceeded 1.0% final concentration and triethylamine never exceeded 0.006% final concentration in this mixture. The amount of inhibitor used was equal to that required to give 100% inhibition of enzyme activity.

Amino-terminal sequencing of proteins. Amino-terminal sequencing was done by gas-phase Edman degradation, using an Applied Biosystems 470A gas-phase sequenator with a reverse-phase C18 column (2.1 by 250 mm) (15).

Immunization and production of hybridoma cell lines. Monoclonal antibodies (MAbs) were prepared as previously described (44). Briefly, 50 μg of protein of the concentrated peak of bactericidal activity from the Bio-Rex 70 column was used to immunize 8-week-old female BALB/cJ mice (Jackson Laboratories, Bar Harbor, Maine) by intraperitoneal injection in Freund's complete adjuvant (GIBCO Laboratories, Grand Island, N.Y.). Two subsequent subcutaneous immunizations of 50 μg of protein in Freund's incomplete adjuvant at 2-week intervals were followed by a fourth intraperitoneal immunization of 50 μg of protein in phosphate-buffered saline (pH 7.2). Four days later, spleen cells

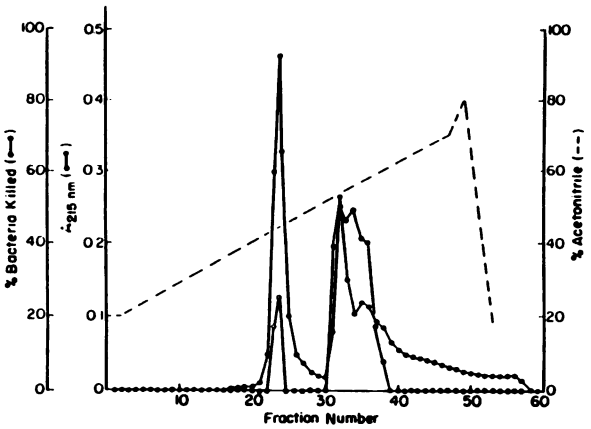

FIG. 1. Bactericidal activity of proteins purified by reverse-phase HPLC. The bactericidal activity and A_{215} of fractions eluted from a C8 reverse-phase column are shown. Approximately 300 μg of protein from the pooled and concentrated bactericidal peak from Bio-Rex 70 was applied to the column.

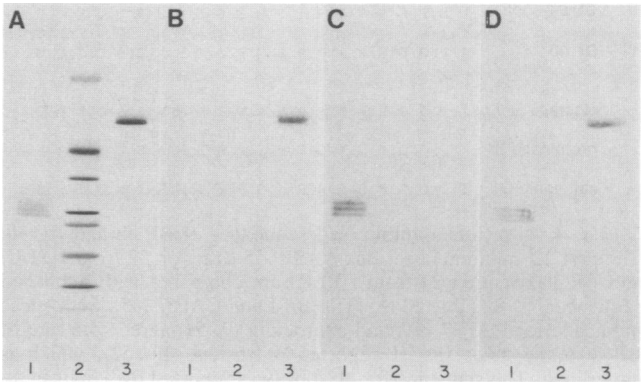

FIG. 2. SDS-PAGE and Western blot analyses of BP 55 and BP 30. In all panels, lane 1 contains 1 μg of protein from fraction 24 from the HPLC profile seen in Fig. 1; lane 2 contains molecular mass standards (bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde 3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; soybean trypsin inhibitor, 20 kDa; α-lactoglobulin, 14 kDa); and lane 3 contains 1 μg of protein from fraction 32 from the HPLC profile seen in Fig. 1. (A) Silver-stained gel after SDS-PAGE. (B to D) Western blot analyses following electrophoretic transfer of proteins onto nitrocellulose. In panel B, MAb AHN-13 is specific for BP 55 and reacts with both bands of the doublet; in panel C, MAb AHN-14.1 is specific for BP 30 and reacts with all three bands; in panel D, MAb AHN-15 reacts with both BP 55 and BP 30. Samples for panels C and D were electrophoresed in a nonreducing SDS-PAGE.

were fused with murine myeloma P3-X63-Ag8.653 cells at a ratio of four spleen cells to one myeloma cell. Hybridoma cell cultures secreting antibodies positive for reaction with proteins of the pooled and concentrated bactericidal fractions from Bio-Rex 70 were determined by radioimmunoassay (44) and were cloned twice in soft agar as described previously (44). Western immunoblot analysis was done by using alkaline phosphatase-labeled goat anti-mouse immunoglobulin M and immunoglobulin G antibody (Boehringer Mannheim) by the method of Blake et al. (4). In some cases, prior to developing the Western blot, periodate oxidation of the transferred proteins was carried out by the method of Woodward et al. (53). The nitrocellulose was incubated in the dark for 1 h at room temperature in 20 mM Tris-HCl (pH 7.5) containing 0.5 M NaCl and 40 mM sodium periodate. This was followed by a 30-min incubation in Tris-NaCl containing 0.133 M glycine; then the steps for the Western blot were performed (4).

RESULTS

Purification of bactericidal proteins. A 55-kDa bactericidal protein (BP 55) was purified by dye-ligand affinity chromatography with Matrex Gel Orange A followed by cation-exchange chromatography with Bio-Rex 70 as previously described (20). This purification scheme yielded a peak of bactericidal activity which, when pooled in its entirety, concentrated, and subjected to reverse-phase high-pressure liquid chromatography (HPLC), resolved into two peaks of bactericidal activity toward *P. aeruginosa*. The bactericidal activity coincided with the two major peaks of absorbance at 215 nm (Fig. 1). Figure 2A shows SDS-PAGE analysis of these two peaks of protein and demonstrates that the first peak, which eluted at 42% acetonitrile (lane 1), contains three bands with an average molecular mass of 30 kDa (BP 30) whereas the second peak, which eluted at 50% acetonitrile (lane 3), contains two proteins with an average molecular mass of 55 kDa (BP 55). The multiple bands of BP 30 were resolved into a single band of 25 kDa when denatured BP 30 was treated with glycopeptidase F (data not shown), as was reported by Campanelli et al. for azurocidin (6). Identical treatment of BP 55 with glycopeptidase F resolved the two forms into one band of approximately 55 kDa (data not shown). SDS-PAGE of each fraction of the bactericidal peak eluted from the Bio-Rex 70 column (Fig. 3) revealed that BP 30 first appeared in the trailing edge of the single bactericidal peak which can be seen in our previous work (Fig. 2 of reference 20). As shown there, the majority of the bactericidal activity and BP 55 eluted from Bio-Rex 70 between fractions 59 and 64 (20), whereas we found BP 30 between fractions 68 and 84.

Characterization of bactericidal proteins. N-terminal amino acid sequences of BP 55 and BP 30 are presented in Fig. 4.

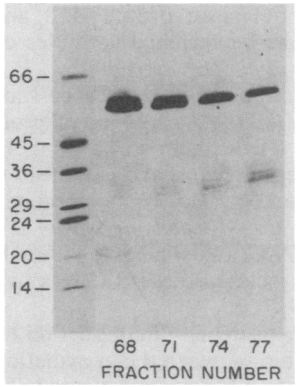

FIG. 3. SDS-PAGE analysis of selected bactericidal fractions eluted from a Bio-Rex 70 cation-exchange column. (Left) Molecular mass standards in kilodaltons (see the legend to Fig. 2). (Right) Fractions from the Bio-Rex 70 column. The profile of bactericidal activity as seen in our previous report (20) showed that most of the bactericidal activity eluted was associated with BP 55 in fractions 59 to 64 and trailed through fraction 77. BP 30 appeared after the major peak of bactericidal activity (fractions 68 to 84). Both BP 55 and BP 30 are seen in fractions 68 to 77 of the silver-stained gel.

BP 55	V N P G V V V R I S Q K G L D Y A S Q Q G R*
BP 30	I V G G R K A R P R Q F P F L A S I Q N Q G
elastase	I V G G R R A R P H A W P F M V S L Q L R G
proteinase 3	I V G G H E A Q P H S R P Y M A S L E M
cathepsin G	I I G G R E S R P H S R P Y M A Y L Q I Q S

FIG. 4. N-terminal amino acid sequences of BP 55 and BP 30. The first 22 amino acid residues of BP 55 (fraction 32 from HPLC) and BP 30 (fraction 24 from HPLC) are compared with published sequences. *, Residue 22 is a T in B/PI and CAP57 (45). Sequences published for CAP37 (31) and azurocidin (9, 52) agree with that of BP 30 except for the H reported at residue 10 (52), which is underlined. The sequence for elastase was reported by Travis et al. (49). The sequence for peak VII (proteinase 3) was reported by Gabay et al. (9); our analysis (34) shows a Q at residue 19 (underlined). The sequence for cathepsin G was reported by Heck et al. (14) and completed by Salvesen et al. (36).

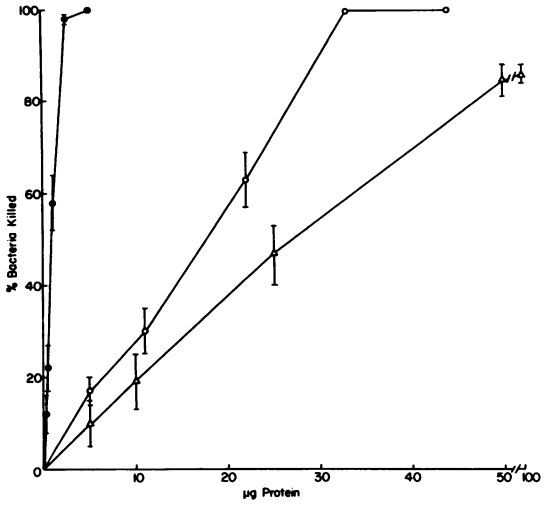

FIG. 5. Comparative bactericidal activities of BP 55 (●), BP 30 (○), and cathepsin G (△) against *P. aeruginosa*. Each value shown is the mean ± one standard error from three determinations.

The amino-terminal sequences for elastase (43, 46, 49), cathepsin G (14, 36, 49), and proteinase 3 are also provided in Fig. 4 for comparison. The sequence for proteinase 3 is that reported by Gabay et al. (9) for the protein eluted in peak VII from an HPLC column. We have also purified proteinase 3 (34) and have confirmed this sequence, with the exception noted in Fig. 4. The amino-terminal sequence for BP 55 agrees with that published for B/PI (30) and CAP57 (45). Our 22-residue sequence for BP 30 agrees with the 20 residues reported for azurocidin by Gabay et al. (9) and with the sequence reported for CAP37 by Pereira et al. (31). The first five amino acid residues of BP 30 are identical to those of elastase, and there is a striking similarity between the amino-terminal sequences of the three serine proteinases of PMNL granules.

The observed sequence similarity of BP 30 and the three serine proteinases led us to include the three proteinases in a study of comparative bactericidal activity. Bactericidal activities of BP 55, BP 30, elastase, proteinase 3, and cathepsin G were compared by using native proteins purified as described in Materials and Methods. *P. aeruginosa* was resistant to killing by up to 100 µg of protein per 5×10^6 bacteria per ml for elastase, proteinase 3, and chymotrypsin. Figure 5 shows the bactericidal activities of BP 55, BP 30, and cathepsin G toward *P. aeruginosa*. *P. aeruginosa* was sensitive to all three proteins, but BP 55 was the most active (50% lethal dose [LD_{50}] = 0.23 µg of protein per 5×10^6 bacteria per ml). BP 30, with an LD_{50} of 16.9 µg of protein per 5×10^6 bacteria per ml, and cathepsin G, with an LD_{50} of 28.3 µg of protein per 5×10^6 bacteria per ml, were much less active. Killing of *P. aeruginosa* by BP 30 isolated by reverse-phase HPLC was similar to killing by native BP 30. However, BP 55 isolated by HPLC was fivefold less active than was native BP 55.

Effects of serine proteinase inhibitors on bactericidal activity. The relationship between the enzymatic and bactericidal activities of cathepsin G was investigated. Table 1 shows the effect of serine proteinase inhibitors on the bactericidal activity of cathepsin G, BP 30, and BP 55 toward *P. aeruginosa*. Proteins were preincubated with phenylmethylsulfonyl fluoride, a general inhibitor of serine proteinase activity; Z-Gly-Leu-Phe-CH_2Cl, a specific chloromethyl ketone inhibitor of cathepsin G; and methoxysuccinyl-Ala-Ala-Pro-Val-CH_2Cl, a specific inhibitor of elastase. Z-Gly-Leu-Phe-CH_2Cl and phenylmethylsulfonyl fluoride inhibited the bactericidal activity of cathepsin G but had little effect on that of either BP 30 or BP 55. The inhibitor specific for elastase had no effect on any of the three bactericidal proteins. The enzyme activity of cathepsin G was measured spectrophotometrically with the specific substrate Suc-Ala-Ala-Pro-Phe-pNA at pH 7.0 and was 100% inhibited at the concentration of the specific and group-specific inhibitors shown in Table 1. Cathepsin G activity was only 20% of the activity at pH 7.0 when the spectrophotometric enzyme assay was carried out at pH 5.6 (results not shown). Enzyme activity was inhibited by phenylmethylsulfonyl fluoride or Z-Gly-Leu-Phe-CH_2Cl at the lower pH. The LD_{50} of cathepsin G for *P. aeruginosa* was the same at pH 5.6 and pH 7.0 (results not shown).

Interaction of serine proteinases with LPS from *P. aeruginosa*. We investigated the possibility that the active site of cathepsin G binds to the bacterial cell surface by studying the influence of free LPS from *P. aeruginosa* ATCC 27312 on enzyme and bactericidal activity toward *P. aeruginosa* type I. Both type I and ATCC 27312 are smooth strains (42).

TABLE 1. Inhibition of bactericidal activity of cathepsin G, BP 55, and BP 30 by serine proteinase inhibitors

Inhibitor	% inhibition (mean ± SE, n = 3) of bactericidal activity[a]		
	Cathepsin G	BP 30	BP 55
Z-Gly-Leu-Phe-CH_2Cl[b] (3×10^{-4} M)	100.0	0.0	2.3 ± 0.1
Phenylmethylsulfonyl fluoride[c] (1×10^{-3} M)	89.5 ± 5.0	1.0 ± 0.2	0.0
MeOSuc-Ala_2-Pro-Val-CH_2Cl[b] (5×10^{-4} M)	1.1 ± 0.4	3.8 ± 0.2	4.3 ± 1.1

[a] One bactericidal unit of BP 55, BP 30, and cathepsin G was preincubated with the inhibitor for 15 min at room temperature before the addition of 5×10^6 *P. aeruginosa* cells.

[b] Z-Gly-Leu-Phe-CH_2Cl is a specific inhibitor of cathepsin G activity, and methoxysuccinyl (MeOSuc)-Ala-Ala-Pro-Val-CH_2Cl is a specific inhibitor of elastase activity.

[c] Phenylmethylsulfonyl fluoride is a general inhibitor of serine proteinase activity.

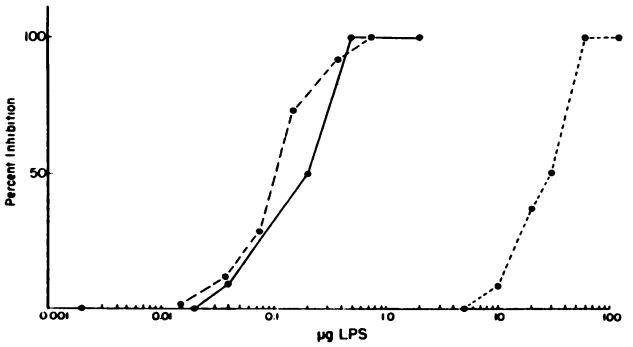

FIG. 6. Percent inhibition of the bactericidal activity of BP 55, BP 30, and cathepsin G by LPS from *P. aeruginosa* 27312. One bactericidal unit of BP 55 (– – –), BP 30 (—), and cathepsin G (----) was preincubated at room temperature with LPS for 15 min before the addition of 5×10^6 *P. aeruginosa* cells.

The effect of free LPS on the bactericidal activity of BP 55 and BP 30 was also investigated. LPS inhibited the bactericidal activity of BP 55, BP 30, and cathepsin G toward *P. aeruginosa*, as seen in Fig. 6. The 50% inhibitory doses calculated by linear regression were 0.153, 0.232, and 30.59 μg of LPS for the inhibition of BP 55, BP 30, and cathepsin G, respectively. When the effect of LPS on the enzymatic activity of cathepsin G was evaluated, LPS from *P. aeruginosa* was found to be a competitive inhibitor of the enzyme activity, as seen in Fig. 7. In contrast, LPS from *Salmonella*

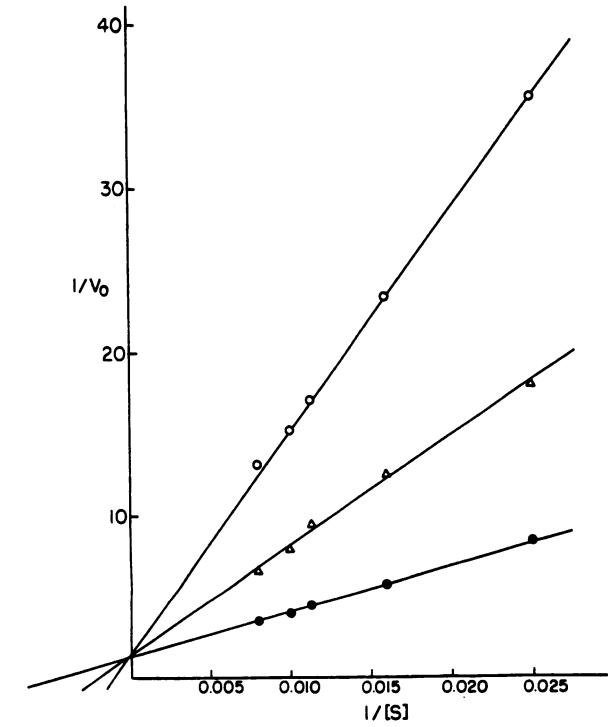

FIG. 7. Lineweaver-Burk plot of the interaction of cathepsin G and LPS from *P. aeruginosa* 27312. Cathepsin G (10 μg) was incubated for 15 min at room temperature with 0 μg (●), 8 μg (△), or 12 μg (○) of LPS before the addition of the different concentrations of the specific substrate for cathepsin G.

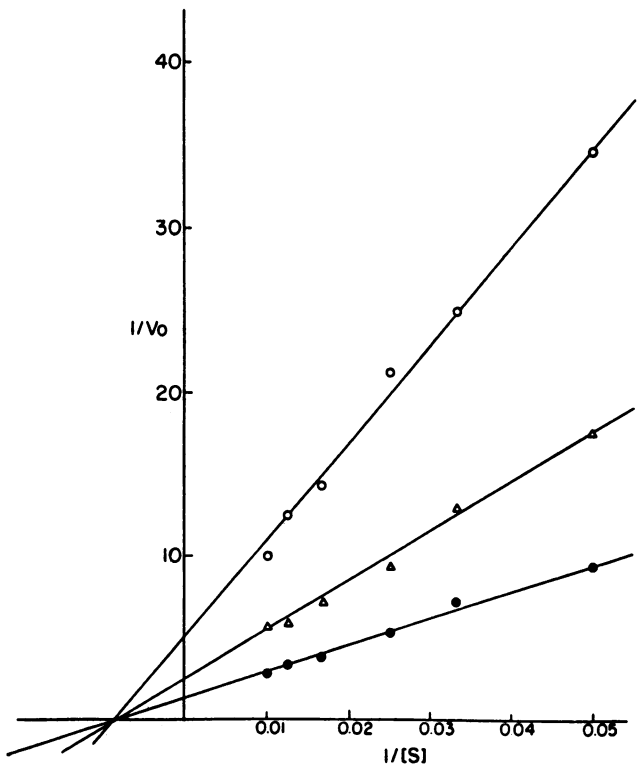

FIG. 8. Lineweaver-Burk plot of the interaction of elastase and LPS from *P. aeruginosa* 27312. Elastase (2 μg) was incubated for 15 min at room temperature with 0 μg (●), 4 μg (△), or 6 μg (○) of LPS before the addition of the different concentrations of the specific substrate for elastase.

minnesota was a noncompetitive inhibitor of cathepsin G (results not shown), suggesting that the interaction of cathepsin G with the LPS of *P. aeruginosa* is unique.

LPS from *P. aeruginosa* was a noncompetitive inhibitor of the enzymatic activity of elastase, as seen in Fig. 8. The enzymatic activities of proteinase 3 and chymotrypsin were not inhibited by 40 μg of LPS from *P. aeruginosa* (results not shown).

Western blot analysis of BP 55 and BP 30. Figure 2B to D shows Western blot analyses for BP 30 and BP 55 with MAbs generated to the concentrated peak of bactericidal fractions from Bio-Rex 70. Figure 2B shows one of the two murine anti-human neutrophil (AHN) MAbs, AHN-13 and AHN-13.1, specific for BP 55. These MAbs reacted with both bands of BP 55 transferred after SDS-PAGE conducted with a sample buffer which contained 2-mercaptoethanol (reducing conditions) and one which did not (nonreducing conditions). The relative mobilities of BP 55 and BP 30 were unchanged when SDS-PAGE was performed under nonreducing conditions. Figure 2C shows one of four MAbs specific for BP 30 (AHN-14, AHN-14.1, AHN-14.2, and AHN-14.3), which reacted with all three bands of BP 30 when SDS-PAGE was performed under nonreducing conditions. The reaction of these MAbs was destroyed when reducing conditions were used for SDS-PAGE of BP 30. The reactivity of the MAb seen in Fig. 2D (AHN-15) was with both BP 55 and BP 30 after nonreducing SDS-PAGE. AHN-15 reacted equally well with BP 55 separated in a reducing gel but did not react with BP 30 when reducing conditions were used for gel electrophoresis.

The possibility that AHN-15 reacts with a common carbohydrate epitope of BP 55 and BP 30 was explored by treating the transblot with sodium *m*-periodate (53). Oxidation of the glycoproteins did not interfere with the Western blot reaction, and, in fact, AHN-15 stained BP 30 more intensely than before periodate treatment (results not shown). The reactivity of none of the other MAbs was diminished by periodate oxidation, and MAbs specific for BP 30 reacted somewhat better with periodate-treated protein than with untreated protein. The estimated molecular mass, the reactivity with the MAbs, and the effects of periodate oxidation were unchanged when the samples applied to the SDS-PAGE were of native protein, rather than HPLC-purified BP 55 or BP 30.

DISCUSSION

Human PMNL are capable of killing *P. aeruginosa* by an oxygen-independent mechanism (19, 20). In the present study, three bactericidal proteins were purified from the granules of PMNL, and all are candidates for a role in the microbicidal mechanism. When compared in terms of their bactericidal activity toward *P. aeruginosa*, BP 55 was 70-fold more active than BP 30 and 120-fold more active than cathepsin G. We also found that the N-terminal 21 amino acid residues of BP 55 were identical to those of B/PI protein (11, 30) and the 57-kDa cationic antimicrobial protein (CAP57) (32, 45), which have been studied with *S. typhimurium* and *E. coli* as targets. The extended sequence of CAP57 (45) and the deduced sequence from a cDNA for B/PI (11) have a T instead of an R at residue 22, which we identified. Despite this discrepancy, there seems little doubt that BP 55, CAP57, and B/PI are the same protein. BP 55 migrated as a doublet when subjected to SDS-PAGE, as reported also for B/PI (26). We have previously shown that BP 55 is a glycoprotein (20), and it is likely that the two molecular forms are due to differences in glycosylation. This possibility is supported both by our identification of antibodies which are specific for BP 55 (AHN-13 and AHN-13.1) and react with both bands by Western blot and by our observation that treatment with glycopeptidase F results in the resolution of a single band upon SDS-PAGE.

Ooi et al. have reported the purification of an active N-terminal 25-kDa fragment of B/PI after reverse-phase HPLC separation of B/PI stored at 4°C (30). We found no evidence of a similar bactericidal fragment of BP 55 in any fraction from our HPLC columns when *P. aeruginosa* was used as the target organism. We did, however, find a bactericidal fraction (BP 30) which eluted prior to BP 55 and migrated as three closely spaced bands of approximately 30 kDa by SDS-PAGE. Inspection of the N-terminal amino acid sequence of BP 30 immediately suggested that it belongs to the family of serine proteinases (12, 24). The N terminus of BP 30 shows 59% similarity with elastase, 45% similarity with cathepsin G, and 47% similarity with proteinase 3 over the first 22 residues. Moreover, of the 29 amino acid differences between BP 30 and the three serine proteinases, 20 (69%) could be the result of a point mutation in the DNA. Shortly after our characterization of BP 30, N-terminal amino acid sequences for CAP37 (31) and azurocidin (9) appeared. Sequence comparison strongly indicates that BP 30, CAP37, and azurocidin are the same protein. The LD_{50} of BP 30 was very high (16.9 μg of protein per 5×10^6 bacteria per ml), but this compares favorably with the 10 μg of protein per 5×10^6 *E. coli* K-12 bacteria reported by Campanelli et al. (6). Studies of this protein indicate that despite the sequence similarity with serine proteinases, enzyme activity is absent by several criteria. First, we have shown (unpublished data), as have Campanelli et al. (6) and Pereira et al. (31), that BP 30 (azurocidin, CAP37) does not act on a variety of peptide and organic ester substrates of serine proteinases. Second, azurocidin (6) and CAP37 (31) fail to bind [^{3}H]diisopropylfluorophosphate (DFP), which is bound by other serine proteinases. The failure to bind [^{3}H]DFP appears to be because there is a glycine residue in the position of the active-site serine residue (52) and a serine residue in the position corresponding to the active-site histidine residue (31). As in the case of BP 55, it is likely that the multiple bands of BP 30 (CAP37, azurocidin) are the result of differential glycosylation. Evidence for this includes the single unambiguous N-terminal amino acid sequence obtained and the reactivity of specific MAbs for BP 30 (AHN-14, AHN-14.1, AHN-14.2, and AHN-14.3) with all three bands. In addition, the multiple bands of BP 30 (azurocidin) were resolved into a single 25-kDa band by treatment with glycopeptidase F (6).

The full-length cDNA and predicted amino acid sequence for B/PI (11) does not contain a stretch of amino acid sequence similarity with BP 30, CAP37, and azurocidin (33). Therefore, BP 55 and BP 30 appear to be two distinct proteins. However, our finding that MAb AHN-15 reacts with both BP 55 and BP 30 suggests that a similar epitope exists in the two proteins and that the proteins might be related. The characteristics of the reactivity of AHN-15 with BP 30 compared with BP 55 also argue against a relationship of primary structure. AHN-15 reacted with BP 30 only under nonreducing conditions, suggesting a role for intramolecular disulfide bonds in the stabilization of the epitope. In contrast, AHN-15 reacted equally well with BP 55 under reducing and nonreducing conditions, demonstrating that disulfide bonds are not necessary to preserve the epitope on BP 55.

When the ability of the three serine proteinases (elastase, cathepsin G, and proteinase 3) to kill *P. aeruginosa* was compared with that of BP 55 and BP 30, only cathepsin G was active and then only at relatively high concentrations (LD_{50} = 28.3 μg of protein). This value is 2.4-fold lower than the 67.5 μg of cathepsin G required to kill 5×10^6 *E. coli* K-12 bacteria reported by others (6). Preincubation of cathepsin G with a group-specific serine proteinase inhibitor or with a cathepsin G-specific chloromethyl ketone inhibitor abolished its bactericidal activity. Preincubation of cathepsin G with LPS from *P. aeruginosa* also inhibited enzyme activity and blocked bactericidal activity. The inhibition of cathepsin G by LPS was competitive, but we do not yet know whether LPS is bound exclusively at the active site or whether this binding orientation is repeated when cathepsin G is bound to intact bacteria. It is clear that the active site must be available for bacterial killing to occur. It does not necessarily follow that the enzymatic activity of cathepsin G is responsible for the bactericidal mechanism at either the binding or the killing step. A comparison of enzyme and bactericidal activity at pH 7.0 versus pH 5.6 showed that the cleavage of the peptide substrate at pH 7.0 was five times greater than at pH 5.6 but that the bactericidal activity was unchanged at the higher pH. This at least suggests that hydrolysis of a bacterial target is not directly involved in killing of *P. aeruginosa* by cathepsin G. Nevertheless, an intact active site is required for killing, and this result is in sharp contrast to an earlier study with *P. aeruginosa* (28), which showed that the bactericidal activity of cathepsin G was not abolished by heat inactivation of the enzyme. The bactericidal activity of cathepsin G toward *Neisseria gonor-*

rhoeae (39, 41), *E. coli* (28), *Staphylococcus aureus* (28, 38), and *Listeria monocytogenes* (1) is also reported to be independent of its proteinase activity, from studies with heat-inactivated (1, 28, 39) and DFP-treated (38, 41) protein. Two peptides of cathepsin G which retain bactericidal activity toward *N. gonorrhoeae* have been isolated (3). One bactericidal peptide corresponds to the five N-terminal amino acids of cathepsin G but is unlikely to be involved in killing by the intact molecule because of evidence that the isoleucine of the N terminus forms an ion pair with the aspartic acid immediately adjacent to the active-site serine residue (35). This would place the peptide in the active site of cathepsin G and raises the possibility that the five N-terminal residues of cathepsin G are involved in killing of *P. aeruginosa*.

Because of the accumulated evidence that bacteria resistant to killing by BP 55 and B/PI do not bind the protein or bind it poorly (18, 50), we have previously assumed that the binding step of the reaction of bacteria with bactericidal protein is a limiting one. However, we have shown that elastase enzyme activity was noncompetitively inhibited by LPS from *P. aeruginosa* but was not bactericidal. Elastase has an unusual hydrophobic site which binds fatty acids and results in noncompetitive inhibition of the enzyme (2), and a hydrophobic interaction of the lipid A moiety of LPS with this site may occur. LPS did not inhibit proteinase 3 activity, but LPS binding at a site remote from the active site remains a strong possibility since the enzyme is reported to bind the LPS from *S. typhimurium* (8). Assays of the direct binding of elastase or proteinase 3 to *P. aeruginosa* will show whether these nonbactericidal proteins are, nevertheless, bound to the bacterial surface. Although BP 55, BP 30, and cathepsin G were each active in killing of *P. aeruginosa*, BP 55 is far more active than either of the others and is therefore more likely to account for the oxygen-independent killing of this microorganism by PMNL. It is therefore surprising that the bactericidal effects of BP 55 and BP 30 are inhibited at approximately the same concentration of LPS. Direct binding studies comparing the affinity of these proteins for binding to bacteria may clarify the relationship of the binding and killing events. Future work which focuses on the structure of BP 55 as it relates to its bactericidal function toward *P. aeruginosa* should enhance our understanding of the microbicidal mechanisms of human PMNL.

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ADDENDUM IN PROOF

Since this paper was submitted, another group has shown that the bactericidal activity of cathepsin G toward *Capnocytophaga sputigena* requires an intact enzyme active site but its bactericidal activity toward *E. coli* does not (K. T. Miyasaki and A. L. Bodeau, J. Clin. Invest. **87**:1585–1593, 1991).

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